FULL CONTENTS

[Claim(s)]
[Claim 1] How to be the transformation method of a monocotyledonous plant and include the process infected in an unhurt seed with the Agrobacterium containing a desired recombination gene.
[Claim 2] The way according to claim 1 said seed is a seed on four to the 5th after seeding.
[Claim 3] The way according to claim 1 or 2 said seed is a germination seed.
[Claim 4] A method given in either of Claim 1 to 3 said whose monocotyledonous plant is a grass.
[Claim 5] The way according to claim 4 said grass is a rice.

[Detailed Description of the Invention]
[0001]
[Field of the Invention] This invention relates to the transformation method of the Agrobacterium medium nature of a monocotyledonous plant.
[0002]
[Description of the Prior Art] A "transformation method" is mentioned as one of the means for improving a plant, and the recombination gene of the request for changing a characteristic is introduced into a plant. An efficient quick transformation method sets a useful plant and the grain which is food especially important as the staple food to carry out molecular breeding, and is very important.
[0003] Many of grain (for example, a rice, wheat, a barley, and corn) is classified into a monocotyledonous plant. In order to carry out the transformation of the monocotyledonous plant, various transformation methods are developed until now. A transformation method is roughly divided into a direct transformation method and an indirect transformation method.
[0004] as a direct transformation method -- for example, the electroporation method (Shimamoto K. et al. --) Nature, 338:274-276, 1989;, and Rhodes C.A. et al., Science, 240:204-207, and 1989 -- reference and the party Kurgan method (Christou P. et al. --) Bio/Technology Reference and the polyethylene glycol (PEG) method (see Datta, S.K. et al., Bio/Technology, 8:736-740, and 1990) are mentioned in 9:957-962 and 1991. Generally the electroporation method and the party Kurgan method have been used, in order to consider it as the method of introducing a gene comparatively efficiently and to carry out the transformation of the monocotyledonous plant.
[0005] As an indirect transformation method, the transformation method (it may be hereafter called an

Agrobacterium transformation method) of Agrobacterium medium nature is mentioned. An Agrobacterium is a kind of a vegetable pathogenic bacterium. When an Agrobacterium is infected with a plant, it has the character which includes in a plant the T-DNA field which exists on the plasmid (for example, a Ti plasmid or a Ri plasmid) which oneself has. By an Agrobacterium transformation method, the integration to the plant of this T-DNA field is used as a means for introducing a gene into a plant. A plant is briefly infected with a desired recombination gene **** Agrobacterium. After infection, a desired recombination gene is introduced in a plant cell from an Agrobacterium, and is included in a plant genome.

[0006] The Agrobacterium transformation method is fully established about the dicotyledonous plant, and many stable transformation plants which will discover a desired recombination gene by the present are created.

[0007] By contrast, generally, it has been conventionally made difficult to apply an Agrobacterium transformation method to a monocotyledonous plant. For example, Portrykus and others (BIO/TECHNOLOGY, 535-542, 1990) has reported not infecting an Agrobacterium with a monocotyledonous plant. However, a possibility that many trials which carry out the transformation of the monocotyledonous plant using an Agrobacterium are performed on the other hand, and an Agrobacterium transformation method can be applied to a monocotyledonous plant as a result has been found out.

[0008] For example, Raineri and others took out the scutellum portion of the rice, gave the crack, planted it in the medium which guides dedifferentiation, and was infected with the Agrobacterium in the scutellum portion several days afterward. As a result, although it did not result by the time it obtained the normal redifferentiation individual, it succeeded in guiding Kars where the foreign gene was introduced (see Raineri, D.M. et al., Bio/Technology, 8:33-38, and 1990).

[0009] The International-Publication WO 94/No. 00977 pamphlet indicates the Agrobacterium transformation method about a rice and corn. or [that it is in dedifferentiation process as a vegetable sample for carrying out a transformation with an Agrobacterium by this method] -- or it needs to use the engineered tissue (for example, Kars) which dedifferentiated. For this reason, in order to produce the engineered tissue where dedifferentiation was carried out from the vegetable sample (for example, leaf section) which is going to carry out a transformation before infection with an Agrobacterium, the dedifferentiation induction period for three to four weeks is usually needed.

[0010] a rice and the corn of the International-Publication WO 95/No. 06722 pamphlet are unripe -- the method infected with an Agrobacterium in an embryo is indicated. however, unripe -- the work for taking out an embryo requires a great labor.

[0011] Therefore, if the quicker and efficient Agrobacterium transformation method of a monocotyledonous plant can be used, it can greatly contribute to the molecular breeding of a useful monocotyledonous plant containing grain, such as a rice.

[0012]

[Problem to be solved by the invention] This invention means solution of the above-mentioned technical problem. The purpose of this invention is to offer the improvement in the Agrobacterium transformation method of a monocotyledonous plant. According to the method of this invention, it is more efficient than the conventional Agrobacterium transformation method, and it is far possible quickly to create a transformation plant.

[0013]

[Means for solving problem] About the transformation method of a monocotyledonous plant, this invention is the Agrobacterium containing a desired recombination gene, and includes the process infected in an unhurt seed. In the method of this invention, processing of a seed being infected in the unhurt status and dedifferentiating the vegetable sample which is going to carry out a transformation is not needed.

[0014] The seed with which infection with an Agrobacterium is presented may be a seed on four to the 5th after seeding. Moreover, a seed may be in the status which budded at the time of infection.

[0015] The monocotyledonous plant by which a transformation is carried out is a grass preferably, and is a rice (Oryza sativa L.) more preferably.

[0016]

[Mode for carrying out the invention] This invention is explained in detail hereafter.

[0017] The "plant" to which the method of this invention is applied is a monocotyledonous plant. As a desirable monocotyledonous plant, a grass (for example, a rice and corn) is mentioned. The most desirable plant to which the method of this invention is applied is a rice, and is a japonica rice especially. Moreover, especially a "plant" means the seed obtained from a plant body and a plant body, unless others show.

[0018] (Production of the vector for a vegetable manifestation) In order to introduce a desired recombination gene into a monocotyledonous plant, the suitable vector for a vegetable manifestation containing a desired recombination gene is built. Such a vector for a vegetable manifestation may be produced by the person skilled in the art using well-known gene modification technology. Although the vector of a pBI system is suitably used for construction of the vector for a vegetable manifestation for using it in an Agrobacterium transformation method, for example, it is not limited to these.

[0019] That "a desired recombination gene" is introduced into a plant says the arbitrary polynucleotides for which it asks. The recombination gene of the request in this invention is not limited to what was isolated from nature, but may also contain a synthetic polynucleotide. Arrangement may obtain a synthetic polynucleotide by compounding or changing a well-known gene with the well-known procedure to a person skilled in the art, for example. As a recombination gene of the request in this invention For example, the arbitrary polynucleotides by which it asks for a manifestation in the plant by which a transformation is carried out and which are endogenicity or exogenism to the plant, And the polynucleotide including the antisense arrangement of the gene which serves as the target in the case of asking for manifestation control of the endogenous gene set for the plant is mentioned.

[0020] When a manifestation is meant in a plant, [a desired recombination gene] [include / in the style which can operate / a self promotor (namely, promotor who has connected this gene possible / an operation / in nature)] Or when a self promotor is not included, or when to include promotors other than a self promotor further is wished, it is connected possible [arbitrary suitable promotors and operations]. As a promotor who may be used, a composition promotor, the promotor who is alternatively discovered in some plant bodies, and an inductive promotor are mentioned.

[0021] In the vector for a vegetable manifestation, it may be connected in the status that further various regulation elements can operate in the cell of a host plant. A regulation element may contain a selection marker gene, a vegetable promotor, a terminator, and an enhancer suitably. It is the matter of the common knowledge to a person skilled in the art that the type of the vector for a vegetable manifestation and the kind of regulation element which are used may change according to the purpose of a transformation.

[0022] A "selection marker gene" may be used in order to make selection of a transformation plant easy. The hygromycin phosphotransferase (HPT) gene for giving hygromycin tolerance, And although a drug resistance gene like the neomycin phosphotransferase II (NPTII) gene for giving a kanamycin resistant may be used suitably, it is not limited to these.
[0023] A "vegetable promotor" means the promotor who is connected with a selection marker gene possible [an operation] and who is discovered with a plant. Although 35S promotor of a cauliflower mosaic virus (CaMV) and the promotor of nopaline synthesis enzyme are mentioned as such a promotor's example, it is not limited to these.
[0024] A "terminator" is arrangement which participates in the conclusion of the transfer at the time of being located downstream from the field which carries out the code of the protein of a gene, and DNA being transferred by mRNA, and addition of poly A arrangement. Although CaMV35S terminator and the terminator (Tnos) of a nopaline synthesis enzyme gene are mentioned as an example of a terminator, it is not limited to these.
[0025] An "enhancer" may be used in order to raise the manifestation efficiency of an objective gene. As an enhancer, the enhancer field including the arrangement of the upstream in CaMV35S promotor is suitable. Two or more enhancers per vector for a vegetable manifestation may be used.
[0026] (Vegetable transformation) The Agrobacteriums used for the transformation of a monocotyledonous plant may be arbitrary Agrobacterium bacteria, and are Agrobacterium(s) preferably. It is tumefaciens. The transformation of the Agrobacterium is carried out by the vector for a vegetable manifestation containing a desired recombination gene (for example, electroporation). A desired recombination gene can be introduced into a plant by infecting a seed with the Agrobacterium by which the transformation was carried out. The introduced recombination gene is included in the genome in a plant, and exists. In addition, it is called the genome in a plant including the genome contained not only in a nuclear staining object but in the various organelles in a plant cell (for example, a mitochondrion, a chloroplast, etc.).
[0027] After removing chaff, preculture of the vegetable seed with which a transformation is meant is carried out unhurt status. It says that "flawlessness" is in the status that the seed has not received artificial operation of removing an ovule, damaging a scutellum about a seed.
[0028] In preculture, seeding of the seed is carried out to the medium (for example, N6D medium) containing auxin (for example, 2, 4-D) of suitable concentration, and it may be typically kept warm for five days preferably during the 4th to the 5th. Preculture is completed before the organization of a seed goes into dedifferentiation process. 25-35 degrees C of temperature at this time is 27-32 degrees C preferably typically. After completion of preculture, a seed is sterilized and, subsequently is fully washed with water. Subsequently, a seed may be infected with the Agrobacterium by which the transformation was carried out under aseptic manipulation.
[0029] A seed is typically kept warm for three days preferably for two days - for five days under dark during infection (cocultivation) with an Agrobacterium. 26-28 degrees C of temperature at this time is 28 degrees C preferably typically. Subsequently, in order to disinfect the Agrobacterium in a medium, processing by a suitable disinfection agent (for example, carbenicillin) is presented with a seed. The seed by which the transformation was carried out is selected on the basis of a selection marker (for example, drug tolerances, such as hygromycin tolerance).
[0030] after cultivating under relevant disinfection conditions and selection conditions, the selected transformation seed is moved to the redifferentiation medium (for example, MS medium) containing a

suitable plant regulator -- a suitable period -- it may be kept warm. In order to rework a plant body, the redifferentiated transformant is moved to a rooting medium (for example, MS medium which does not contain a plant regulator). After growth of a root is checked, the bowl raising of the transformant may be carried out.

[0031] The recombination gene of the request introduced into the plant can act for the purpose (for example, control of the manifestation of a new characteristic made into the purpose, or a certain endogenous gene expression) meant in a plant.

[0032] It may be checked by the person skilled in the art using the well-known procedure whether the desired recombination gene has been introduced into the plant. This check can be performed using northern blot analysis, for example. All the RNA is specifically extracted from the leaf of the reworked plant, and a blot is carried out to a suitable membrane after the electrophoresis in denaturation agarose. mRNA of the target gene can be detected by making this blot hybridize a part of transgene and the complementary RNA probe which carried out the sign. Or by introduction of a desired recombination gene, when asking for manifestation control of the endogenous gene in a plant, the manifestation of the endogenous gene which serves as a target can be examined, for example using the above-mentioned northern blot analysis. When the manifestation of the endogenous gene which serves as a target is intentionally controlled compared with the manifestation in the control plant of a non-transformation, a desired recombination gene is introduced into a plant and having acted on control of a manifestation is checked.

[0033] The conventional method usually needs the dedifferentiation induction period for three to four weeks, before infection with an Agrobacterium. By contrast, since the method of this invention does not need the process which guides dedifferentiation, it can shorten days required in order to create a transformation monocotyledonous plant. Furthermore, according to the method of this invention, it also becomes possible to shorten the period which the selection in a conventional method takes, and it becomes possible [reducing the influence of cultivation variation].

[0034] In one embodiment with the desirable method of this invention, the days needed in order to create a transformation monocotyledonous plant are about 50 days, and are about 2/3 or less [of the days (about 90 days) needed in the conventional Agrobacterium transformation method (see the following work example 2)]. Moreover, according to the method of this invention, in the case of a fine [Japanese] seed, the transformation efficiency of 10 to 15% is acquired. Other rice kinds, such as deep KITAAKE, can attain transformation efficiency high to the same extent with a bang. Therefore, it is more possible than the conventional transformation method by using the method of this invention to create a transformation plant efficiently and quickly.

[0035]

[Working example] A work example is given to below and this invention is explained concretely. This work example does not limit this invention. Material, a reagent, etc. which were used in the work example are available from a commercial source of supply, as long as there is no other specification.

[0036] (Work example 1: Transformation of the rice plant by the method of this invention) The seed fine [Japanese] which is the typical kind of a rice was sterilized in 2.5% sodium hypochlorite (NaClO) solution in the state of [unhurt] after removal of chaff. After water washed [sufficient], the following aseptic manipulation was presented with the rice.

[0037] (Preculture) Seeding of the seed was carried out to the N6D medium (a 30g/l sucrose, 0.3g/l casamino acids, a 2.8g/l proline, 2mg/l 2, 4-D, 4g/l Gherla Ito, pH 5.8) containing 2 and 4-D, and it was

kept warm at 27 degrees C - 32 degrees C for five days. The seed budded in the meantime (drawing 1).

[0038] (Vector for a vegetable manifestation) as a vector for a vegetable manifestation for carrying out the transformation of the Agrobacterium pIG121Hm which is the plasmid with which the GUS gene containing the 1st intron of the catalase gene of Ricinus communis and the hygromycin resistance gene were connected was used (Nakamura et al., the vegetable biotechnology II, a modernization study special number, pp.123-132 (1991)). The transformation of Agrobacterium EHA101 was carried out by pIG121Hm (Hood et al., J. Bacteriol., 168:1291-1301 (1986)). EHA101, the vir field of a helper plasmid is the bacillus of the strong virulence Agrobacterium A281 origin.

[0039] (Agrobacterium infection) [the suspension of the Agrobacterium by which the transformation was carried out] After the above-mentioned seed which carried out preculture was immersed, it transplanted to the 2N6-AS medium (a 30g/l sucrose, 10g/l glucose, 0.3g/l casamino acids, 2mg/l 2, 4-D, 10 mg/l aceto SHIRINGON, 4g/l Gherla Ito, pH 5.2). Cocultivation was kept warm and carried out at 28 degrees C for three days under dark.

[0040] (Disinfection and selection) The Agrobacterium was flushed from the seed after completion of cocultivation using the N6D medium containing 500 mg/l carbenicillin. Subsequently, the seed by which the transformation was carried out was selected on condition of the following.

1st selection: The seed was placed on the N6D medium containing 2 of 2 mg/l which filled up carbenicillin (500 mg/l) and hygromycin (25 mg/l), and 4-D, and it was kept warm at 27 degrees C - 32 degrees C for seven days.

2nd selection: The seed was placed on the N6D medium containing 2 of 2 - 4 mg/l which filled up carbenicillin (500 mg/l) and hygromycin (25 mg/l), and 4-D, and it was kept warm at 27 degrees C - 32 degrees C for seven more days.

[0041] (Redifferentiation) Redifferentiation of the selected transformation seed was carried out on condition of the following.

1st redifferentiation: -- a redifferentiation medium (MS medium (a 30g/l sucrose --) which filled up carbenicillin (500 mg/l) and hygromycin (25 mg/l)) A 30g/l sorbitol, 2g/l casamino acids, 2 mg/l kinetin, 0.002 mg/l The picked seed was put on NAA, 4g/l Gherla Ito, and pH 5.8 top, and it was kept warm at 27 degrees C - 32 degrees C for two weeks.

2nd redifferentiation: The same redifferentiation medium as having used it in the 1st redifferentiation was used, and it was kept warm at 27 degrees C - 32 degrees C for further two weeks.

[0042] (Bowl raising) The bowl raising was carried out, after moving the redifferentiated transformant on the rooting medium (MS medium which filled up hygromycin (25 mg/l) and which does not contain hormone) and checking growth of a root (drawing 2).

[0043] (Work example 2: Transformation of the rice plant by the conventional method) For comparison with the method of a description, a Japanese fine one was used for the work example 1 as a material of a transformation, and the transformation of the rice plant by the conventional method was performed as follows.

[0044] (Kars guidance) After removal of chaff, it sterilized, and seeding of the seed fine [Japanese] was carried out to the Kars inducer medium (2 of 2 mg/l, N6D medium containing 4-D), and this was kept warm at 30 degrees C under the bright place. Kars which the scutellum origin increased was used for the transformation about four weeks after the Kars guidance start.

[0045] (Transformation) By Agrobacterium EHA101 which carried out the transformation by vector pIG121Hm for a vegetable manifestation so that it might be indicated in the work example 1, it was

infected, and it was kept warm in three days on the 2N6-AS medium, was kept warm at 28 degrees C under dark, and cocultivation of obtained Kars was carried out.
[0046] (Disinfection and selection) The Agrobacterium was flushed from Kars using the N6D medium containing 500 mg/l carbenicillin. Subsequently, Kars by which the transformation was carried out was selected on condition of the following.
1st selection: Kars was placed on the N6D medium containing 2 of 2 mg/l which filled up carbenicillin (500 mg/l) and hygromycin (50 mg/l), and 4-D, and it was kept warm at 27 degrees C - 32 degrees C for two weeks.
2nd selection: Kars was placed on the N6D medium containing 2 of 2 which filled up carbenicillin (500 mg/l) and hygromycin (50 mg/l) - 4 mg/l, and 4-D, and it was kept warm at 27 degrees C - 32 degrees C for further two weeks.
[0047] (Redifferentiation, rooting, and bowl raising) Redifferentiation of the selected transformation seed was carried out on the same conditions as a work example 1, and even the bowl raising was performed.
[0048] (Result) Comparison with the example of the transformation by the conventional method and the example of the transformation by the method of this invention is shown in drawing 3 . Days required by the bowl raising of a transformant were [in / to having been about 90 days / the method of this invention] about 50 days in the conventional method after seeding (drawing 3 (a)). When compared after seeding as of the 50th [about] day, the transformant in a conventional method still suited the process of redifferentiation to the transformant in the method of this invention having been in the status in which a bowl raising is possible (drawing 3 (b)). When collected, the period which a transformation takes was shortened by operation of the method of this invention about 2/3 or less [of a conventional method].
[0049]
[Effect of the Invention] According to this invention, the transformation method of the monocotyledonous plant of the improved Agrobacterium medium nature is offered. In the method of this invention, the unhurt seed of the plant which has a transformation meant is infected with the Agrobacterium containing a desired recombination gene. Use of this invention enables it to create a transformation plant more quickly more efficiently.

[Brief Description of the Drawings]
[Drawing 1] It is the photograph in which the status of a rice seed just before an Agrobacterium is infected is shown and in which the form of a living thing is shown.
[Drawing 2] it is the photograph in which the redifferentiation individual of the rice which could come out from seeding by the method of this invention for the 50th [about] day is shown and in which the form of a living thing is shown.
[Drawing 3] (a) It is the photograph which compared the transformant by the conventional method for the 90th [about] day with the transformant according to the method of this invention for the 50th [about] day after seeding and in which the form of a living thing is shown after seeding. (b) It is the photograph which compared the transformant by the conventional method for the 50th [about] day with the transformant according to the method of this invention for the 50th [about] day after seeding and in

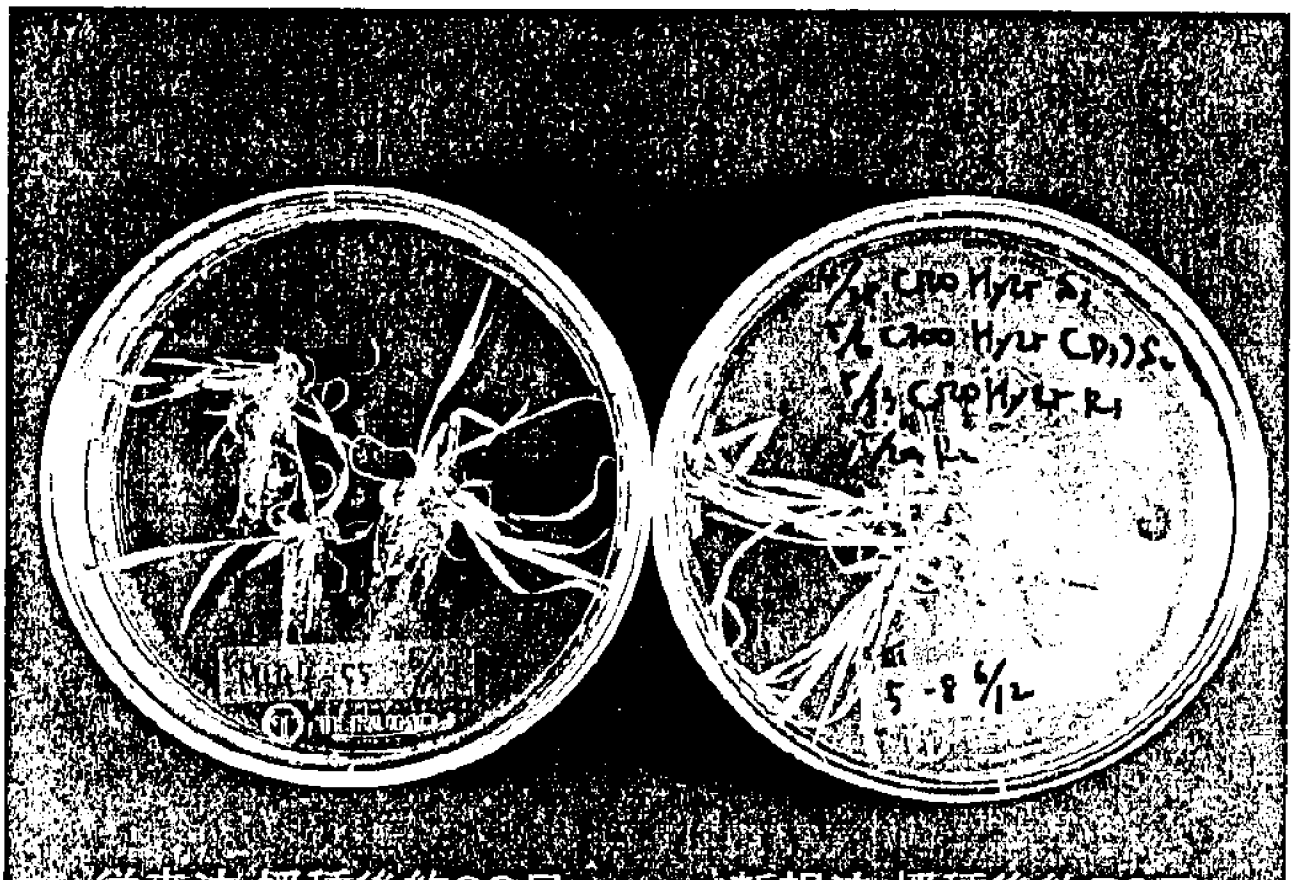
which the form of a living thing is shown after seeding.

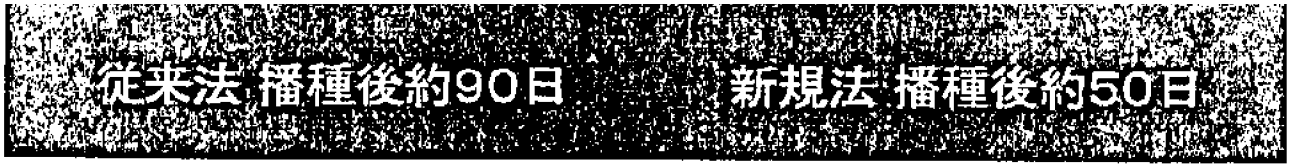
[Drawing 1]

[Drawing 2]

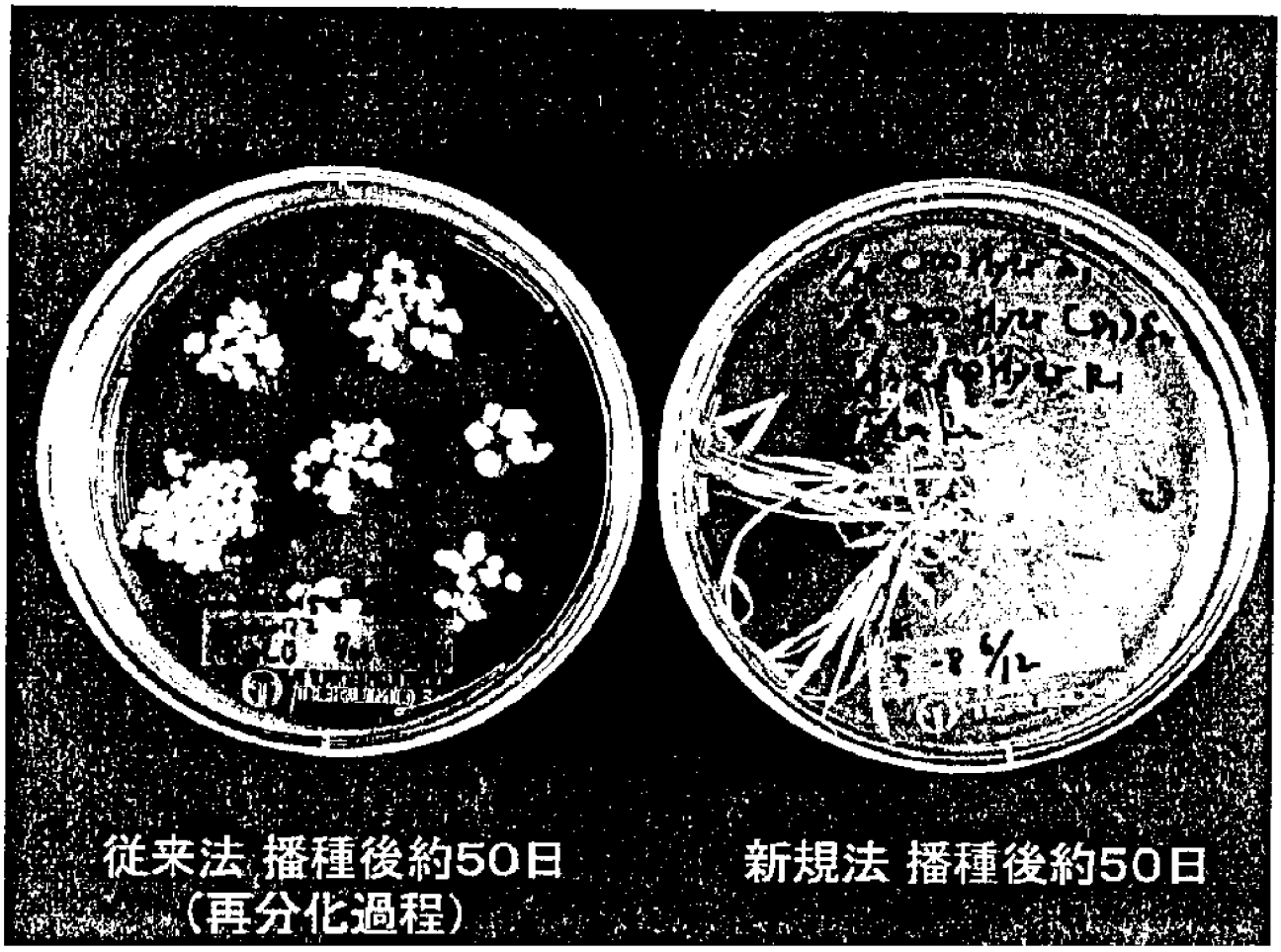
[Drawing 3]

(a)





(b)



[A Written Amendment]
[Filing Date] Heisei 12(2000) February 25 (2000. 2.25)
[Amendment 1]
[Document to be Amended] Description
[Item(s) to be Amended] Claims
[Method of Amendment] Change
[Proposed Amendment]
[Claim(s)]
[Claim 1] It is the way it is the transformation method of a monocotyledonous plant, the process infected in an unhurt seed with the Agrobacterium containing a desired recombination gene is included, and this

seed is a germination seed here.
[Claim 2] The way according to claim 1 said seed is a seed on four to the 5th after seeding.
[Claim 3] The way according to claim 1 or 2 said monocotyledonous plant is a grass.
[Claim 4] The way according to claim 3 said grass is a rice.

[Translation done.]